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Pistacia lentiscus leaves as a source of phenolic compounds: Microwave-assisted extraction optimized and compared with ultrasound-assisted and conventional solvent extraction

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ABSTRACT

Microwave assisted extraction (MAE) was investigated for extraction of total phenolic compounds (TPC expressed as gallic acid equivalents GAE) from the leaves of *Pistacia lentiscus* L. with maximized total phenolic yield using response surface methodology (RSM) coupled with a Box–Behnken design. The optimal MAE processing parameters were 46% ethanol, extraction time 60 s, potency density 17.86 W/mL, and liquid/solid ratio 28:1, with an extraction yield of $185.69 \pm 18.35\ mg_{GAE}/g_{dw}$. The optimized MAE was compared with another emerging technology (ultrasound assisted extraction, UAE) and with conventional solvent extraction (CSE) giving higher extraction yields of TPC, total flavonoids and tannins and comparable antioxidant capacity (according to the radical ABTS assay).

1. Introduction

Pistacia lentiscus L. (lentisk) is an evergreen bush that can reach 3 m in high, belonging to the Anacardiaceae family that consists of more than eleven species. It is largely distributed in "extreme" ecosystems of the Mediterranean countries and has a large geographical and bioclimatical distribution, extending from the humid to the arid areas (Lo Presti et al., 2008).

In Algeria, the tree is widespread in forest alone or associated with other tree species such as terebinth, olives and carob, in all coastal areas up to 700 m above sea level or in seaside stony areas. It succeeds in any ordinary garden soil, preferring a hot dry position in full sun. It prefers a well-drained to dry sandy or stony alkaline soil, making it more abundant near the sea. It also shows tolerance to rocky areas, drought and cold (−7 °C) in winter together with resistance to calcareous soil and re-growth after cutting fire injuries (Yildirim, 2012).

Aqueous extract of *P. lentiscus* L. leaves is a very popular drink in North Africa countries and is becoming increasingly popular worldwide, partly because of more documented evidence about its beneficial health properties (Trabelsi et al., 2012). *P. lentiscus* L. is a rich source of essential oils (96 components) (Lo Presti et al., 2008), fatty acids such as oleic, palmitic and linoleic acids (representing the 50.72%, 23.16% and 21.75% of the lipid fraction, respectively) and polyphenols (Trabelsi et al., 2012). The latter represent the 7.5% of leaf dry weight and include myricetin glucuronide, myricetin 3-O-rutinoside and myricetin 3-O-rhamnoside (20% of total phenolic content), quercetin 3-O-rhamnoside, delphinidin 3-O-glucoside, cyanidine 3-O-glucoside, phenolic acids such as gallic acid (3.7 mg/g dry weight) and 5-O-galloyl quinic acid (9.6 mg/g dry weight) (Romani et al., 2002). *P. lentiscus* L. has a great nutritional and industrial importance, particularly in the pharmaceutical industry, and the current interest in the health effects of lentisk has stimulated the development of new extraction processes of phenolic compounds fractions. Indeed, the quality of polyphenol extracts and their antioxidant capacity depends not only on the quality of the starting biomass (geographic origin, climatic conditions, harvesting date and storage conditions), but also on the technological processes involved in their manufacture (Nkhili et al., 2009).

Furthermore, a recent work (Gratani et al., 2013) has estimated the potential benefit to the global carbon cycle that would

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come from extending the Mediterranean shrub lands thanks to the good yearly carbon dioxide sequestration of some analysed species, including *P. lentiscus* L. Restoration projects and reintroduction actions of native species, in particular in more degraded or dryer areas in the Mediterranean region, could then increase the availability of biological residues associated to these plantations, therefore remunerative and sustainable processes for their valorization are looked for, such as the recovery of antioxidant substances.

Over the past decade, various novel extraction techniques have been introduced and investigated, most of which were claimed to improve efficiency, extract quality, extraction time and solvent consumption. The emerging techniques available are microwave assisted extraction (MAE) (Song et al., 2011), ultrasound assisted extraction (UAE) (Carrera et al., 2011), supercritical fluid extraction (SFE) (Santos et al., 2012) and pressurized solvent extraction (Xiao et al., 2012). MAE has particularly drawn significant research attention in various fields, such as recovering of active ingredients from plant materials. One of the main advantages of MAE is the significant reduction of processing time (from several hours to minutes) compared to conventional solvent liquid extraction (CSE) and a high yield of active substances (Chan et al., 2011).

As MAE is influenced by many factors including irradiation time, potency density, temperature, type of solvent as well as the interactions of all these factors, a statistical optimization needs to be adopted for determination of the optimum operating conditions. Response surface methodology (RSM) is a statistical method that uses quantitative data from an appropriate experimental design to determine or simultaneously solve multivariate equation. RSM can then generate a mathematical model and take into account the possible interrelationships among the test variables while minimising the number of experiments (Song et al., 2011) which is particularly useful in an experimental design with more than two factors allowing for considerable reduction of running cost and time (Cheok et al., 2012). In this study, a Box–Behnken design (BBD) was adopted for the experimental planning since BBD is one of the most efficient designs of experiment methods. An advantage of the BBD is that it does not contain combinations for which all factors are simultaneously at their highest or lowest levels. BBD is then useful in avoiding experiments performed under extreme conditions, for which unsatisfactory results are often obtained.

To the best of our knowledge, no literature report exists on the optimization of MAE procedure for the extraction of total phenolic compounds (TPC) from *P. lentiscus* L. leaves. Therefore, the objectives of the current study were to:

- investigate the effects of different parameters on the extraction efficiency (in terms of TPC recovery) by MAE process through preliminary single-factor experiments;
- optimize the MAE conditions by RSM;
- compare the optimized MAE process with CSE process and with UAE as another emerging non-conventional process (in terms of TPC, flavonoids, condensed tannins recovery, antioxidant capacity of the recovered compounds, and process effect on tissue damage).

Furthermore, since antioxidant compounds represent, usually, a minor fraction of the biomass, an integral exploitation of the residue after extraction should be looked for, according to a biorefinery approach. A general chemical characterization of the biomass was, then, carried out to evaluate the fibre content. Lignocellulose fractionation, in fact, could be proposed to recover the three main fractions hemicellulose, cellulose and lignin.

Finally, for a more complete evaluation of MAE implementation as a potential green technology, calculation of its energy consumption compared to CSE and UAE was also addressed.

2. Materials and methods

2.1. Plant material

The leaves of *P. lentiscus* L. were harvested in June 2012 from spontaneous plants in Oued Ghir, Bejaia, located in the North East of Algeria. The collected samples were identified by the Vegetable Ecological Laboratory and the voucher specimens have been deposited at the Herbarium of the Algiers University, Algeria. The samples were washed with tap water and distilled water, dried in a static oven at 40 °C for about one week, and then ground in an electrical grinder (IKA model A_{11} Basic). The powder was passed through standard 125 μm sieve and stored in airtight bags under darkness until use. Moisture content was assessed by constant weight at 105 °C and was 5.0 ± 0.5%. Content in ethanol–toluene extractives, lignin, holocellulose, cellulose and hemicelluloses, given on an oven dry weight basis, was determined according to the methods reported by Amendola et al. (2012).

2.2. Reagents

Sodium carbonate (Na_2CO_3), Folin–Ciocalteu's phenol reagent, disodium hydrogen phosphate (Na_2HPO_4), hydrochloric acid (HCl) and aluminium chloride ($AlCl_3{\cdot}6H_2O$) were obtained from Prolabo (CE); 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), polyvinyl polypyrrolidone (PVPP) and $FeSO_4$ from Sigma–Aldrich (Germany), and gallic acid from Biochem-chemopharma (UK). All solvents used were of analytical grade and purchased from Prolabo (CE).

2.3. Experimental work

For optimization of the MAE procedure, the influences of the process parameters were firstly separately investigated in single-factor experiments to limit the total experimental work (Table 1). When one variable was not studied, it was kept constant. The constant values for irradiation time, solvent-to-solid ratio (S/S ratio), ethanol concentration and microwave power were 120 s, 20 mL/g, 50% and 500 W, respectively. Since in all the trials the leaves powder amount was kept constant at 1 g, the S/S ratio gives the mL of solvent and can be used to calculate the power density (W/mL) which, as a consequence, varied both in the microwave power experiments and in the S/S ratio experiments. On the basis of the single-factor experimental results, major influence factors were selected.

For both the single-factor experiments and the successive RSM optimization, only the TPC yield was considered. In fact, since the Folin assay used to quantify TPC (see Section 2.4.1) actually measures the reducing power of the compounds, the results are influenced by the oxidative status of the sample and are, then, related to the antioxidant quality of the extract.

Then, an RSM based on a Box–Behnken design (BBD) was conducted to optimize the processes (Table 2). A three-level-four-factor BBD experimental design was applied and the number of experiments (N) required was defined according to Eq. (1):

$$N = 2k(k-1) + C_0 \quad (1)$$

where k is the number of factors and C_0 is the number of central points (3).

Regression analysis of the data to fit a second-order polynomial equation (quadratic model) was carried out according to the

Table 1
Results of single-factor experiments for microwave assisted extraction from *P. lentiscus* leaves. Results are reported as means ± SD. Same letters in the same column refer to means not statistically different according to ANOVA and Tukey's test. TPC, total phenolic compounds; GAE, gallic acid equivalents; dw, dry weight of leaves.

Ethanol concentration		Extraction time		Power density			Solvent-to-solid ratio		
%, v/v	TPC yield (mg_{GAE}/g_{dw})	s	TPC yield (mg_{GAE}/g_{dw})	W	W/mL	TPC yield (mg_{GAE}/g_{dw})	mL/g	W/mL	TPC yield (mg_{GAE}/g_{dw})
20	144.94 ± 2.06^{b}	30	149.39 ± 8.11^{c}	300	15	158.27 ± 4.72^{b}	10	50	82.34 ± 4.30^{c}
40	160.46 ± 4.63^{a}	60	179.22 ± 6.43^{a}	400	20	157.67 ± 4.75^{b}	20	25	163.40 ± 5.64^{ab}
60	154.58 ± 3.41^{ab}	90	168.52 ± 5.13^{ab}	500	25	179.22 ± 6.43^{a}	25	20	171.98 ± 4.49^{a}
80	152.91 ± 4.55^{ab}	120	144.72 ± 4.08^{c}	600	30	166.26 ± 4.76^{ab}	30	16.7	160.80 ± 4.06^{ab}
100	150.14 ± 4.63^{ab}	150	147.28 ± 7.87^{c}	700	35	168.07 ± 5.22^{ab}	40	12.5	152.70 ± 4.98^{b}
		180	160.38 ± 4.17^{bc}	900	45	166.26 ± 5.70^{ab}			
		210	157.37 ± 3.59^{bc}						

following general equation (Eq. (2)) which was, then, used to predict the optimum conditions of extraction process.

$$Y = B_0 + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x^2 + \sum_{i>j}^{k} B_{ij} x_i x_j + E \quad (2)$$

where Y represents the response function (in our case the TPC yield); B_0 is a constant coefficient; B_i, B_{ii} and B_{ij} are the coefficients of the linear, quadratic and interactive terms, respectively, and x_i and x_j represent the coded independent variables. The factor levels were coded as −1 (low), 0 (central point or middle) and 1 (high), respectively. The variables were coded according to the following equation (Eq. (3)):

$$x_i = \frac{X_i - X_0}{\Delta X} \quad (3)$$

where x_i is the (dimensionless) coded value of the variable X_i; X_0 is the value of X at the centre point and ΔX is the step change.

According to the analysis of variance, the regression coefficients of individual linear, quadratic and interaction terms were determined. In order to visualize the effects of independent variables and their mutual interaction, the regression coefficients were used to generate 3-D surface plots from the fitted polynomial equation.

To verify the adequacy of the model, additional extraction trials were carried out at the optimal conditions predicted with the RSM and the obtained experimental data were compared to the values predicted by the regression model.

Optimized MAE conditions were then compared to a reference CSE procedure and to another emerging non-conventional extraction technique (UAE).

Finally, in order to investigate the influence of ultrasound, microwave and simple solvent maceration on the microstructure of the samples powder, the peel powder samples (before and after the different extraction processes) were observed by scanning electron microscopy (SEM).

2.3.1. Microwave-assisted extraction

A domestic microwave oven (NN-S674MF, Samsung, Malaysia) with cavity dimensions of 22.5 cm × 37.5 cm × 38.6 cm and 2450 kHz working frequency was used. The apparatus was equipped with a digital control system for irradiation time and microwave power (the latter was linearly adjustable from 200 to 1000 W). The oven was modified in order to condensate into the sample the vapours generated during extraction.

For extraction, 1 g of *P. lentiscus* L. powder was placed in a round bottom flask of 250 mL (medium neck of 45 mm) containing

Table 2
Box– Behnken design with the observed responses and predicted values for yield of total phenolic compounds (TPC) referred to dry weight (dw) of *P. lentiscus* leaves using microwave assisted extraction. GAE, gallic acid equivalents; S/S, solvent to solid.

Run	X_1 Ethanol concentration (%, v/v)	X_2 Power		X_3 Irradiation time (s)	X_4 S/S ratio (mL/g)	TPC yield (mg_{GAE}/g_{dw})	
		W	W/mL			Experimental	Predicted
1	40	400	10.00	60	40	175.15	171.62
2	60	600	20.00	60	30	163.70	168.41
3	40	400	13.33	90	30	187.96	193.33
4	60	500	16.67	90	30	175.53	174.48
5	60	500	16.67	30	30	164.75	169.77
6	20	600	20.00	60	30	151.12	146.55
7	40	600	30.00	60	20	175.15	177.67
8	40	500	25.00	30	20	171.01	167.49
9	40	500	12.50	30	40	166.86	162.28
10	60	500	12.50	60	40	147.31	141.65
11	20	500	12.50	60	40	114.96	123.57
12	60	500	25.00	60	20	165.38	159.40
13	40	600	15.00	60	40	158.27	161.54
14	20	500	16.66	90	30	156.17	150.15
15	20	500	25.00	60	20	113.38	119.10
16	40	500	12.50	90	40	168.52	170.42
17	40	600	20.00	30	30	185.92	183.17
18	40	500	25.00	90	20	175.52	178.56
19	40	600	20.00	90	30	202.42	199.34
20	40	400	20.00	60	20	173.04	168.80
21	20	400	13.33	60	30	146.15	139.92
22	20	500	16.67	30	30	135.68	135.75
23	40	400	13.33	30	30	184.49	190.50
24	60	400	13.33	60	30	173.34	176.26
25	40	500	16.67	60	30	179.61	182.70
26	40	500	16.67	60	30	176.32	182.70
27	40	500	16.67	60	30	178.41	182.70

water–ethanol mixture at different ethanol concentrations (20, 40, 60, 80, 100% (v/v)). The suspension was irradiated at regular intervals according to oven operation. Depending on the trial, a different solvent, irradiation time, microwave power and solvent-to-solid ratio were used (Tables 1 and 2). At the end of microwave irradiation, the volumetric flask was allowed to cool to room temperature (Dahmoune et al., 2013). After extraction, the extract was recovered by filtration on a Büchner funnel through No. 1 Whatman paper and collected in a volumetric flask. The extract was stored at 4 °C until use and analysed for the TPC. The extract obtained under the optimum conditions by RSM was analysed also for the content of flavonoids, condensed tannins and for the antioxidant capacity.

2.3.2. Ultrasound assisted extraction

An ultrasonic apparatus (SONICS Vibra cell, VCX 130 PB, Stepped microtips and probes, No. 630-0422) was used for UAE with working frequency fixed at 20 kHz. The energy input was controlled by setting the amplitude of the sonicator probe.

For the extraction, 1 g of fine powder was placed in a 250 mL amber glass bottle ($Ø \times H$: 45 mm × 140 mm and cap size of 28 mm) containing water–ethanol mixture; the obtained suspension was exposed to acoustic waves for 15 min. The temperature (27 ± 2 °C) was controlled continuously by circulating external cold water and checking the temperature using a T-type thermocouple (Cooking, Thermo-Timer, China). The acoustic energy density (AED) employed in this experiment was 0.01 W/mL. AED was calculated at an amplitude level of 45 μm, with temperature recorded during 15 min of experiment (Rawson et al., 2011). After the extraction, the extract was recovered and analysed as reported in Section 2.3.1 for the optimized MAE extract.

2.3.3. Conventional solvent extraction

For the conventional solvent extraction, 1 g of fine powder was placed in a conical flask of 250 mL ($Ø \times H$: 51 × 150 mm and cap size of 38 mm), and 50 mL of 60% (v/v) EtOH were added. The mixture was kept in a thermostatic water bath (mod. WNB22, Memmert) with shaking speed of 110 strokes per minute, at 60 °C for 2 h, according to the method recommended by Spigno et al. (2007). The extract was then recovered and analysed as reported in Section 2.3.1 for the optimized MAE extract.

2.4. Analytical determinations

2.4.1. Total phenolic compounds

The content in TPC of the *P. lentiscus* L. leaves extract was determined by Folin's assay as reported in literature (Jaramillo-Flores et al., 2003). Briefly, 100 μL of the extract was mixed with 750 μL of 10% diluted Folin–Ciocalteu reagent. The solutions were mixed and incubated at room temperature for 5 min. After incubation, 750 μL of 7.5% sodium carbonate (Na_2CO_3) solution was added. After incubation at 25 °C for 90 min the absorbance was measured at 725 nm (1 cm optical path) against a blank (made as reported for the sample but with 100 μL of sample solvent) using a Spectro Scan 50 UV-Vis spectrophotometer.

Gallic acid hydrate was used as standard for the calibration curve to express the TPC concentration of the sample as mg/L of gallic acid equivalents (GAE). TP yield was then calculated based on the sample concentration, extract volume and leaves powder dry weight, according to the following equation (Eq. (4)):

$$\text{TP yield} = \frac{\text{mg}_{\text{GAE}}/\text{L} \cdot \text{L}_{\text{Extract}}}{\text{g}_{\text{dw leaves powder}}} \quad (4)$$

2.4.2. Condensed tannins

Condensed tannins (CT) were estimated according to the PVPP method (Ribéreau-Gayon et al., 2000). Briefly, 0.2 g of polyvinylpolypyrrolidone (PVPP) was added to 5 mL of extract (diluted 25 times) and mixed with 15 mL of distilled water (acidified to pH 3 with HCl 12 N). After centrifugation at 4600 × *g* for 10 min, the precipitate was rinsed with 10 mL of distilled water, then mixed with 20 mL of a BuOH–HCl (12 N) (1:1, v:v) mixture containing 150 mg/L of $FeSO_4$. After heating for 30 min in a water bath at 95 °C, the optical density (d_1) at 550 nm was measured in a 1 cm optical path cuvette. The optical density of a control (d_0), prepared under the same conditions but not heated, was also measured. The condensed tannin concentration (CTC) was calculated by the following equation (Eq. (5)):

$$\text{CTC} = 273 \cdot (d_1 - d_0) = \text{mg/L} \quad (5)$$

As reported for total phenols, the yield of condensed tannins was calculated based on the sample concentration, extract volume and leaves powder dry weight as reported in (Eq. (6)):

$$\text{CT yield} = \frac{\text{CTC} \cdot \text{L}_{\text{Extract}}}{\text{g}_{\text{dw leaves powder}}} \quad (6)$$

2.4.3. Total flavonoids

The content of total flavonoids (TF) was estimated by the $AlCl_3$ method (Quettier-Deleu et al., 2000). Briefly, 1 mL of extract was added to 1 mL of 2% methanolic $AlCl_3 \cdot 6H_2O$ incubated for 10 min at room temperature. The absorbance was then read at 415 nm (1 cm optical path). The results were expressed in mg quercetin equivalents/g dry weight of fine powder of *P. lentiscus* ($\text{mg}_{\text{QE}}/\text{g}_{\text{dw}}$).

Quercetin was used as standard for the calibration curve to express the TF concentration of the sample as mg/L of quercetin equivalents (QE). TF yield was then calculated based on the sample concentration, extract volume and leaf powder dry weight, according to the following equation (Eq. (7)):

$$\text{TF yield} = \frac{\text{mg}_{\text{QE}}/\text{L} \cdot \text{L}_{\text{Extract}}}{\text{g}_{\text{dw leaves powder}}} \quad (7)$$

2.4.4. Antioxidant capacity

The antioxidant capacity (AOC) of the *P. lentiscus* L. leaves extracts was assessed by the ABTS assay (Spigno and De Faveri, 2009), which is based on the ability of antioxidants of interacting with the ABTS radical, decreasing its absorbance at 734 nm. Briefly, a radical solution (7 mM ABTS and 2.45 mM potassium persulfate) was prepared and incubated in the dark at room-temperature for 12–16 h before use. This solution was then diluted with ethanol 50% to an absorbance of 0.705 ± 0.02 at 734 nm and equilibrated at 30 °C. Control, blank and extract samples were prepared consisting, respectively, in: 2 mL of radical solution, 2 mL of radical solution mixed with 20 μL of sample solvent and 2 mL of radical solution mixed with 20 μL of extract. Absorbance of the three samples was read after 6 min at 734 nm against ethanol 50% and the AOC was calculated as percentage absorbance decrease according to Eq. (8):

$$\text{AOC} = \frac{A_{\text{Blank}} \cdot A_{\text{Extract}}}{A_{\text{Control}}} \cdot 100 \quad (8)$$

The extract had to be diluted with water in order to have a final A_{Extract} above 0.10. Extract samples were, then, always diluted to have a TPC content in the range 40–310 μg_{GAE}/mL. From data elaboration (AOC plotted versus total phenols concentration), the concentration of TPC required to reach 50% radical inhibition (IC_{50}) was calculated.

2.4.5. Scanning electron microscopy (SEM) analysis

The powder (before and after extraction) was observed under SEM (Quanta 200, FEI company) for morphological characterization (Dahmoune et al., 2013). Four samples of powders (untreated and residues after MAE carried out under optimized conditions, CSE and

UAE) were collected and dried until constant mass in oven at 60 °C before SEM analysis.

Sample particles were fixed on the specific carbon film support, and their shape and surface characters were observed using GSED detector with environmental mode (ESEM).

2.5. Statistical analysis

Each extraction trial and all the analyses were carried out in triplicate (in duplicate the chemical characterization of dry leaves) and all the data in this paper have been reported as means ± SD. Influence of each factor on the TPC yield in the single-factor experiment for the MAE was statistically assessed by ANOVA and Tukey's post hoc test with 95% confidence level.

Data obtained from the BBD trials for the MAE were statistically analysed using ANOVA for the response variable in order to test the model significance and suitability. $p < 0.05$ and $p < 0.01$ were taken as significant and highly significant level, respectively.

The JMP (Version 7.0, SAS) and Design-Expert (Trial version 8.0.7.1) software were used to construct the BBD and to analyse all the results.

Influence of extraction technique (MAE, UAE or CSE) on TPC, TF, CT yields and extract AOC was assessed by univariate ANOVA and Tukey's post hoc test for means discrimination (95% confidence level).

3. Results and discussion

Biomass characterization gave the following composition of dry leaves (on a dry weight basis): 21.21 ± 0.11% ethanol–toluene extractives; 36.07 ± 1.15% lignin like fraction; 25.66 ± 1.37% holocellulose; 10.77 ± 0.58% cellulose and 14.89 ± 0.80% hemicelluloses. This is in agreement with literature, which reports that leaves are tissues highly specialized for carbohydrate synthesis (Chapman et al., 2013). The residual biomass after polyphenols extraction could then be exploited for the fractionation of the fibre components (Spigno et al., 2013).

3.1. Microwave-assisted extraction

3.1.1. Single-factor experiments

The effects of various extraction parameters such as ethanol percentage, irradiation time, microwave power and power density, and S/S ratio on the TPC yield were studied using the one-variable-at-a-time approach.

Regarding the influence of ethanol concentration, selection of extraction solvent is critical as it will determine the amount and type of extracted phenolic compounds. Acetone and aqueous alcohols, particularly ethanol and methanol, are the most commonly employed solvents in phenolic extraction from botanical materials, more than the corresponding mono-component solvent system (Inglett et al., 2010; Spigno et al., 2007). However, organic solvents, such as methanol and acetone, are toxic and not acceptable for foods. The use of ethanol has several advantages over the use of other solvents, including higher extraction efficiency, environmental compatibility and lower toxicity and cost. However, the percentage of ethanol in water as an extraction solvent can affect the extraction efficiency (Tabaraki and Nateghi, 2011; Wu et al., 2011). That is why aqueous ethanol at different percentages was tested in this study.

Table 1 shows that the TPC yield raised significantly only when the concentration of ethanol increased from 20 to 40%. At higher concentrations the yields were statistically the same as that at both 20 and 40%. A similar effect was reported for the extraction of phenolic compounds from other plant sources (Spigno et al., 2007; Li et al., 2012; Pan et al., 2003). This phenomenon could be explained by the fact that with the increase in EtOH concentration, solvent polarity declined and molecular movement decreased, which led to light dissolution of phenolic compounds for the lowering of diffusion coefficient and the decrease of solubility (Yang et al., 2009). Related to polarity and then microwave heating mechanism, the dielectric constant of water is higher than ethanol ($\hat{\varepsilon} = 80.4$ and 24.3, respectively), while the dielectric loss factor is lower (22.8 compared to 8.52 at 25 °C) which indicates a slower microwave energy absorption and a higher penetration depth. Therefore, globally the positive effects of intermediate ethanol concentrations can be attributed to both an increased solubility of phenolic compounds and a reduced heating of the mixture with a limited thermal degradation of the recovered compounds. Due to the analytical principle of the Folin assay used to quantify TPC (as commented in Section 2.3), lower values may suggest that intense heating has caused degradation of the bioactive compounds.

Based on these results, the concentration range 40–60% was selected for the RSM trials and 50% was fixed for the next single-factor experiments on the influence of microwave irradiation time.

As shown in Table 1, a significant increase in extraction efficiency was observed as the extraction time increased from 30 to 60/90 s to be followed by a significant decrease after 120 s. Longer irradiation exposition without temperature control probably induced thermal degradation of phenolic compounds (Yang et al., 2009). Since shorter extraction time is also favourable to reduce energy costs, the 30–90 s range was selected for the RSM trials, while 120 s was kept for the next single-factor trials.

In the evaluation of the effect of a different irradiation power, the same S/S ratio was maintained giving the corresponding power density reported in Table 1. Power density significantly influenced the TPC yield in the tested condition. The yield increased with increasing microwave power from 300 to 500 W and then it remained constant or slightly decreased for higher powers (average values at 600/700/900 W were statistically not different from both values at 400 and 500 W). It must be underlined that the operating temperature could not be regulated in the used equipment and that for a constant sample size temperature increases with microwave power (Spigno and De Faveri, 2009). Since it has been reported that the main effect of microwaves, and in many cases the only, is the heating effect (Kappe et al., 2013), the obtained results were probably due to an enhancement of phenols recovery due to a heating effect, with consequent increase of mass transfer phenomena, up to a certain power density value and, then, to thermal degradation of bioactive compounds at higher densities (Li et al., 2012).

The range 400–600 W was selected for the RSM study, while the 500 W power was used for the last single-factor trials dealing with a varying S/S ratio, which, brought also to have a varying power density. The results at a S/S of 10 confirmed what previously supposed since the power density was higher (50 W/mL) and the TPC yield was very low. Due to the small size of the sample with this S/S, its width was very close or even lower than the microwave penetration depth (a penetration depth of 1.4 and 0.42 cm is reported for water and ethanol respectively at 25 °C (www.pueshner.com; Horikoshi et al., 2009). As already reported by Spigno and De Faveri (2009) this causes significant amounts of electric field reach the back face of the samples and are reflected, so that the electric field in the sample is increased many folds and the solvent heating rate increases dramatically with a consequent thermal degradation of the compounds, which explains the low measured TPC yield. For all the other tested values, the TPC yields were almost non statistically different with an increasing trend for S/S ratio from 20 to 25 and a decreasing trend from 25 to 40. The observed results can be explained by the contemporary variation of the two factors S/S ratio and power density. As also reported by Spigno and De Faveri (2009), the increase in the liquid/solid ratio can lead to increased yield

due to an increased concentration gradient which drives the mass transfer of the solutes from the solvent impregnated on the solid particles into the external solvent (this is the same effect that can be observed in CSE). However, in MAE, when the solvent amount increases also the sample size increases and if the microwave power is kept constant, the power density is reduced and the total sample heating is reduced as well. Since temperature decrease leads to reduction in mass transfer coefficients, at a certain liquid/solid ratio this effect could not be compensated anymore by the increased concentration gradient. This result underlines the importance of considering in microwave heating not only the microwave power but also the sample size when combined with power, determines the more important factor of power density.

The 20–40 S/S ratio range was finally selected for the RSM trials.

3.1.2. Optimization by RSM

Table 2 shows the results of TPC recovery obtained in the BBD experiments and the corresponding predicted values according to the applied second-order regression model.

The mathematical Eq. (9) correlating the recovery of TPC with MAE process variables is given below in terms of coded factors excluding non-significant terms:

$$\text{TPC yield} = 178.11 + 13.85x_1 + 4.26x_3 - 27.46x_1^2 + 5.95x_3^2 - 14.55x_4^2 \quad (9)$$

In order to determine whether the quadratic model is significant, it is necessary to run ANOVA analysis. The results of the second order response surface model fitting in the form of ANOVA are given in Table 3. The ANOVA demonstrated the model to be significant as evident from F value and p value (Yang et al., 2009). The determination coefficient ($R^2 = 0.95$) indicates that only 5% of the total variations is not explained by the model. For a good statistical model, the adjusted determination coefficient R^2_{adj} should be close to R^2. In our model it was 0.90 and then quite close to R^2. Moreover, R^2_{pred} 0.81 is in reasonable agreement with R^2_{adj} and confirms that the model is highly significant. The regression coefficient from experimental data and the adjusted one were reasonably close to 1, which indicated a high degree of correlation between the observed and predicted values. At the same time, a low value of the coefficient of variation indicated a high degree of precision and a good deal of reliability of the experimental values. The lack of fit test determines if the model is adequate to describe the experimental data or whether another model should be reselected. The value of lack of fit test (0.0539) is higher than 0.05 which is not significant relative to the pure error and indicates that the fitting model is adequate to describe the experimental data. An adequate precision is a measure of the signal to noise ratio, which greater than 4 is considered to be desirable (Canettieri et al., 2013). In our model the value of adequate precision was 15.98, demonstrating an adequate signal. At the same time, a relatively low value of coefficient of variation (CV) (2.91) indicates a better precision and reliability. Therefore, the obtained model is adequate for prediction in the range of experimental variables.

The significance of each coefficient measured using p-value and F-value is listed in Table 3. Smaller p-value and greater F-value mean the corresponding variables would be more significant. The p-value of the model is less than 0.0001, which indicates that the model is significant and can be used to optimize the extraction variables.

The effects of the independent variables and their mutual interactions on the TPC yield can be visualized on the three dimensional response surface plots and two dimensions contour plots shown in Figs. 1 and 2, respectively. The plots were generated by plotting the response using the z-axis against two independent variables while keeping the other two independent variables at their zero level (Hayat et al., 2009). Each 3D plot represents the number of combinations of the two-test variable. 3D response surface and 2D contour plots are the graphical representations of regression equation and are very useful to judge the relationship between independent and dependent variables. Different shapes of the contour plots indicate whether the mutual interactions between the variables are significant or not. Circular contour plot means the interactions between the corresponding variables are negligible, while elliptical contour suggests the interactions between the corresponding variables are significant (Liu et al., 2013).

The recovery of TPC mainly depends on the ethanol concentration as its quadratic and linear effects were highly significant ($p < 0.001$), confirming the single-factor experiment results. Extraction of phenolics could be enhanced using an aqueous ethanol over a limited compositional range. This finding was in agreement with our previous conclusions on extraction of natural phenolic compounds from *Citrus limon* peels (Dahmoune et al., 2013). Other literature works have shown the significant influence of the ethanol percentage on the phenolic extraction from plant materials, such as *Rosmarinus officinalis* (Švarc-Gajic et al., 2009) and green tea leaves (Pan et al., 2003). In the latter case, the extraction was increased

Table 3
Analysis of variance (ANOVA) for the experimental results obtained by using microwave assisted extraction.

Source	Sum of squares	Degrees of freedom	Mean square	F-value	p-ValueProb > F
Model	10,293.45	14	735.24	17.41	<0.0001
X_1-Solvent	2303.64	1	2303.64	54.57	<0.0001
X_2-Power	1.04	1	1.04	0.02	0.8776
X_3-Time	217.83	1	217.83	5.16	0.0423
X_4-Ratio	149.97	1	149.97	3.55	0.0839
X_1X_2	53.36	1	53.36	1.26	0.2828
X_1X_3	63.94	1	63.94	1.51	0.2420
X_1X_4	96.50	1	96.50	2.28	0.1564
X_2X_3	42.51	1	42.51	1.01	0.3354
X_2X_4	90.15	1	90.15	2.13	0.1696
X_3X_4	2.04	1	2.04	0.04	0.8295
X_1^2	4024.13	1	4024.13	95.34	< 0.0001
X_2^2	259.10	1	259.10	6.13	0.0291
X_3^2	188.87	1	188.87	4.47	0.0560
X_4^2	1128.61	1	1128.61	26.73	0.0002
Residual	506.49	12	42.20		
Lack of fit	500.91	10	50.09	17.96	0.0539
Pure error	5.57	2	2.79		
Cor total	10,799.95	26			
$R^2 = 0.95$	Adj $R^2 = 0.90$	Pred $R^2 = 0.81$	C.V. % = 2.91		

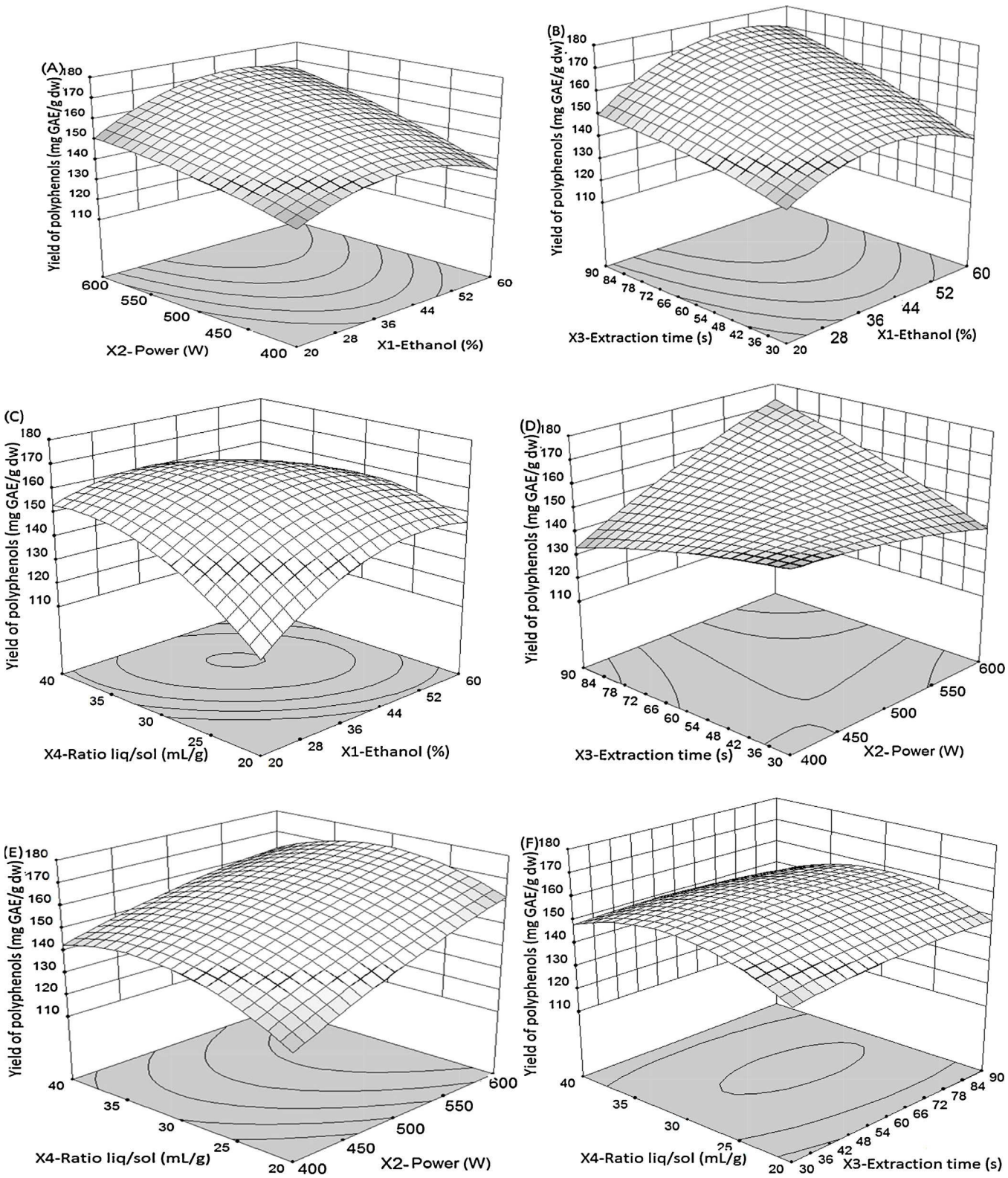

Fig. 1. Response surface analysis for the total phenolic yield from *P. lentiscus* leaves with microwave assisted extraction with respect to ethanol percentage and microwave power (A); irradiation time and ethanol percentage (B); solvent-to-solid ratio and ethanol percentage (C); microwave power and irradiation time (D) solvent-to-solid ratio and microwave power (E) solvent-to-solid ratio and irradiation time (F).

when the proportion of ethanol/water solvent was lower than 50% (v/v) and decreased when higher than 50% (v/v).

None of the interactive terms of the model was significant and Figs. 1 and 2 show that the TPC could be maximized using about 45% ethanol over a range of the other operational factors (microwave power, irradiation time and S/S ratio).

The linear term of irradiation power was significant, while the linear terms for microwave power and S/S ration were not

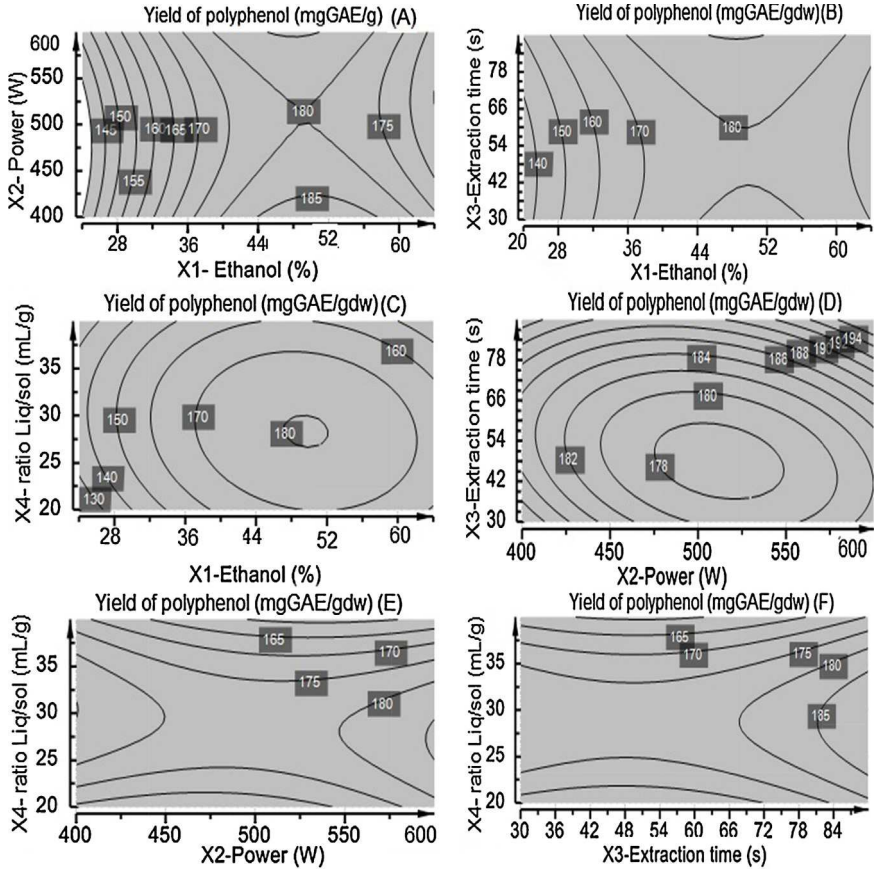

Fig. 2. Contour plots for the total phenolic yield from *P. lentiscus* leaves with microwave assisted extraction with respect to ethanol percentage and microwave power (A); irradiation time and ethanol percentage (B); solvent-to-solid ratio and ethanol percentage (C); microwave power and irradiation time (D) solvent-to-solid ratio and microwave power (E) solvent-to-solid ratio and irradiation time (F).

significant again in agreement with the preliminary trials. This is particularly evident for the microwave power ($p = 0.8776$), since, as previously commented, this parameter should be more properly considered in combination with sample size, that is to say looking at the power density. The quadratic terms were significant for all the variables, except irradiation time.

Also in another literature study on analytical-scale MAE (Wang et al., 2010) it was found that the interactions between microwave powers, extraction time and ethanol proportion were not significant but the tendency was reversed with linear and quadratic effect.

In conclusion, using an adequate ethanol concentration, the other factors can set at desired levels in order to minimize the energy consumption and environmental impact of the process (solvent amount, irradiation power and time) which is fundamental for the potential large scale application of MAE.

Using the derived model (Eq. (9)), the optimal MAE conditions for the TPC yield were obtained: ethanol concentration 46%; irradiation time 60 s; microwave power 500 W and S/S ratio 28 mL/g (corresponding to a power density of 17.86 W/mL). Under optimal conditions, the model predicted a maximum response of $180.43 \pm 18.05\ mg_{GAE}/g_{dw}$.

To validate the predictability of the established model, the optimized parameters were tested in an additional experiment. A mean value of $185.69 \pm 18.35\ mg_{GAE}/g_{dw}$ was obtained which was found to be not significantly different than the predicted one at $p > 0.05$ using a paired t-test (Hossain et al., 2012), confirming that the model was adequate for reflecting the expected optimization (Wang et al., 2010).

3.2. Comparison between MAE, UAE and CSE

The optimized MAE conditions were compared with another emerging extraction technique (UAE) and with a CSE. The operating conditions selected for UAE (15 min, 40% ethanol, energy density 2.6 W/mL and S/S ratio 50) come from not yet published trials on the application of ultrasounds extraction to *P. lentiscus* L. leaves. The parameters adopted in CSE (120 min, 60% ethanol, S/S ratio 50) were taken by the method of Spigno et al. (2007). The ethanol concentration was not exactly the same, but in the MAE single-factor experiments; there was not a highly significant difference between 40 and 60%. In addition, the S/S ratio was lower in the MAE but it must be said that in the UAE and CSE protocols the temperature was kept constant; therefore, a higher S/S ratio should only enhance phenols extraction. Furthermore, since one of the most commonly appreciated advantages for MAE is the reduction in solvent consumption, we preferred to have a lower S/S ratio for this technology.

The results show that the three investigated extraction techniques gave statistically comparable TPC yields (185.69 ± 18.35, 142.76 ± 19.98, $178.00 \pm 19.80\ mg_{GAE}/g_{dw}$ for MAE, UAE and CSE respectively). The TF yield was significantly different for all the three processes and higher with MAE ($5.16 \pm 0.22\ mg_{QE}/g_{dw}$) than with UAE and CSE (4.61 ± 0.02 and $4.79 \pm 0.03\ mg_{QE}/g_{dw}$, respectively). Also the CT yield was significantly higher with MAE ($40.21 \pm 1.76\ mg/g_{dw}$) than with UAE ($35.94 \pm 1.13\ mg/g_{dw}$) or with CSE ($31.15 \pm 3.88\ mg/g_{dw}$).

Different total phenolic yield extraction values from leaves can be found in literature depending on the botanical source and

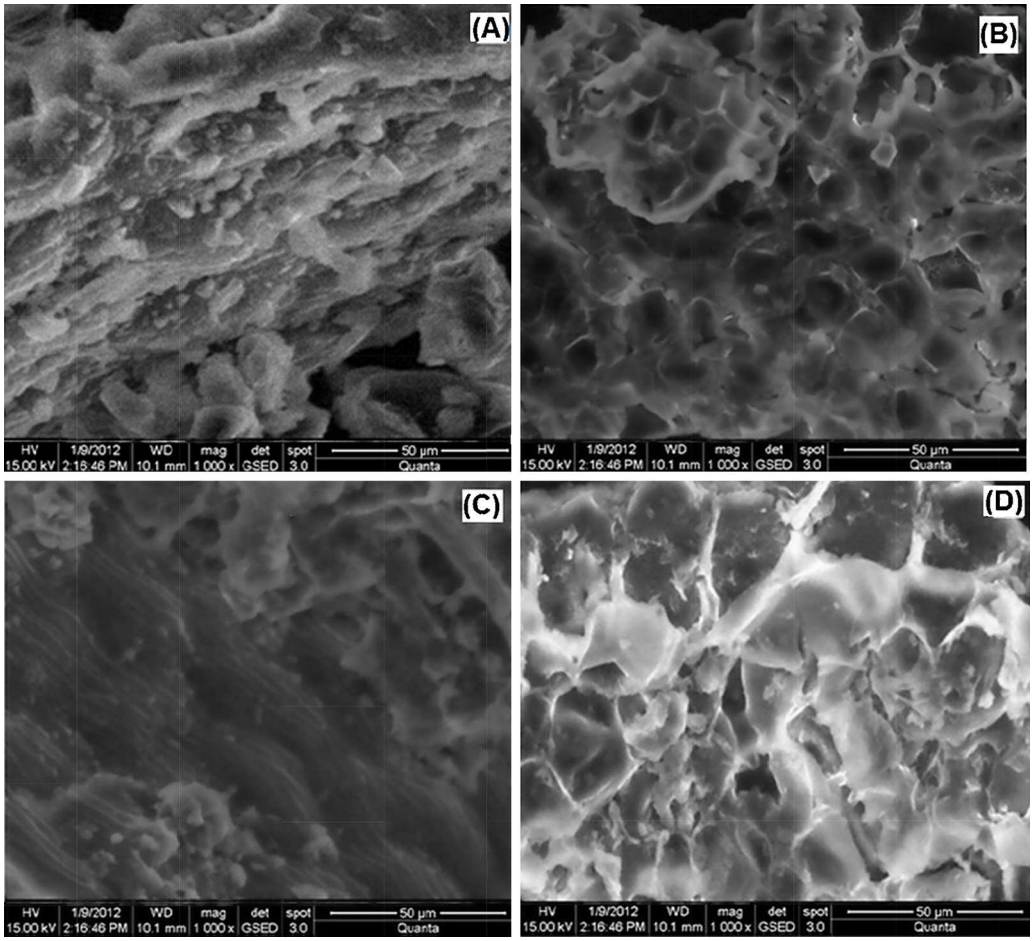

Fig. 3. Scanning electron microscope images of *P. lentiscus* leaves powder before (A) and after extraction by ultrasound assisted extraction (B), conventional solvent extraction (C) and microwave assisted extraction (D).

applied extraction technique and solvent. Karabegović et al. (2014) found, in agreement with the present work, that the extracts from cherry laurel leaves obtained by MAE contained the highest amount of phenolic and flavonoid compounds compared to UAE and CE. They recovered up to 30 mg_{GAE}/g_{dw}. Nour et al. (2014) extracted about 40 mg_{GAE}/g_{dw} of TPC from blackcurrant leaves with 40% aqueous ethanol. Lower TPC yield, 6.2 mg_{GAE}/g_{dw}, was reported by López-Mejía et al. (2014) for the TPC extraction of amaranth leaves.

The AOC of the obtained extract was comparable for MAE and CSE extracts (IC_{50} of 126.19 ± 1.88 and 130.59 ± 1.33 μg_{GAE}/mL, respectively) and higher than UAE extracts (IC_{50} 189.69 ± 2.18 μg_{GAE}/mL). The lower activity of UAE extract could be the result of some ultrasound related effect.

MAE has the advantage of a reduced use of solvent and a shorter extraction time particularly compared to CSE. A rough estimation of energy consumption in the three systems was carried out.

In the case of MAE and UAE, the nominal power densities (17.9 and 2.6 W/mL, respectively) and extraction times were considered together with a solvent volume of 50 mL, obtaining similar energy consumptions: 107.4 and 117 kJ for MAE and UAE, respectively. For CSE the energy needs to heat initially the 50 mL of solvent can be estimated in 6.7 kJ, based on a solvent density of 0.887 g/mL, a solvent specific heat of 3.77 $J g^{-1} {}^{\circ}C^{-1}$ (Perry and Green, 1998) and an initial solvent temperature of 20 °C. However, the efficiency of the used heating system, plus the energy consumptions for sample mixing and temperature maintenance during the 2 h extraction should be also added, therefore it is very difficult an accurate energy costs comparison for the CSE.

Finally, the plant materials treated by MAE, UAE and CSE were examined by SEM to investigate the effect of the different extraction methods on the physical structure of the fine powder (Fig. 3). Obvious fractural changes in the surface morphology became evident after 60 s of microwave irradiation energy (Fig. 3D) and 15 min of ultrasound treatment (Fig. 3B); while no severe fracture was observed during the conventional extraction (Fig. 3C) except few slight ruptures on the surface of the sample. The surface of the sample after MAE was found greatly destroyed suggesting microwave irradiation played an important role in breaking up vegetal cell walls. In addition, this phenomenon suggests that microwave action affects the physical structure of the cell due to the molecular movement and rotation of liquids with a permanent dipole, leading to speedy heating of the ethanol/water solvent. Sudden temperature rise and internal pressure increase is different from that of CSE which depends on a series of permeation and solubilization processes to bring the chemicals out of the matrix. In case of UAE, the swelling and softening process of the cell wall was also observed (Fig. 3B) probably via the hydration of pectinous material from middle lamella, which might lead to the break-up of vegetal tissue during sonication (Ma et al., 2008).

4. Conclusions

This present study indicates that *P. lentiscus* L. leaves can be considered as a good source of phyto-pharmaceutical interest compounds since it was possible to recover up to 18.6% (on dry weigh basis) of total phenolic compounds with antioxidant capacity.

MAE was optimized through a RSM based approach and the optimal determined conditions resulted 46% aqueous ethanol as solvent, 17.86 W/mL as power density, 60 s as irradiation time and 28:1 S/S ratio. The extract obtained under these conditions showed a total phenolic compounds yield comparable to the CSE and UAE extracts, an average 10% higher total flavonoids yield, and a 10–30% higher condensed tannins yield compared to UAE and CSE, respectively. The antioxidant capacity was comparable to that of the CSE extract, but a 34% higher than for the UAE extract.

SEM observation of the extraction residues showed that plant tissue was greatly destroyed after MAE. This probably enhanced the fast diffusion of the compounds into the solvent.

Even though the optimized MAE process allows for definitely shorter working time and lower S/S ratio than CSE and UAE, this does not automatically mean energy savings, since microwave heating needs electrical energy that is its most expensive form, so a punctual energy cost analysis should be required to compare the conventional and MAE extraction systems (a rough energy consumption estimation showed similar values for MAE and UAE but it was not possible to accurately evaluate the value for the CSE).

Anyway, from the point of view of industrial application, this research could be the basis for further pilot-plant trials of MAE as a green extraction technology for the recovery of high-added value compounds from biomass residues. Implementation of the found optimal conditions in batch microwave extractors equipped with mixing device or in continuous microwave applicators, should be investigated.

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